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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 15 August 2002 (15.08.02)	
International application No. PCT/US01/06288	**Applicant's or agent's file reference** 1465-PCT-00
International filing date (day/month/year) 28 February 2001 (28.02.01)	**Priority date** (day/month/year) 01 March 2000 (01.03.00)
Applicant DANIELL, Henry	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

21 September 2001 (21.09.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Christine Carrié
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

[redacted]TENT COOPERATION TREA[redacted]

09/807742

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

WEISER, Gerard, J.
1600 Market Street, Suite 3600
Philadelphia, PA 19103-7286
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 01 November 2001 (01.11.01)	
Applicant's or agent's file reference 1465-PCT-00	**IMPORTANT NOTIFICATION**
International application No. PCT/US01/06288	International filing date (day/month/year) 28 February 2001 (28.02.01)
International publication date (day/month/year) 04 October 2001 (04.10.01)	Priority date (day/month/year) 01 March 2000 (01.03.00)
Applicant AUBURN UNIVERSITY et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
01 Marc 2000 (01.03.00)	60/185,987	US	11 Sept 2001 (11.09.01)
23 Janu 2001 (23.01.01)	60/263,424	US	16 Octo 2001 (16.10.01)
23 Janu 2001 (23.01.01)	60/263,473	US	21 Sept 2001 (21.09.01)
23 Janu 2001 (23.01.01)	60/263,668	US	21 Sept 2001 (21.09.01)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
Carlos NARANJO

Telephone No. (41-22) 338.83.38

INTERNATIONAL SEARCH REPORT

International application No
PCT/US01/06288

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 15/14, 15/16, 15/17, 15/20, 15 21, 15 31, 15 32, 15/62, 15 82, C12P 21 02, A01H 5/00
US CL : 435/320.1, 70.1, 418; 530 412; 536/23.4, 23.5, 23.51, 23.52, 23 7, 23 71; 800/278, 279, 288, 298, 301, 317.3

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/320.1, 70.1, 418; 530/412; 536/23.4, 23.5, 23.51, 23.52, 23.7, 23.71; 800/278, 279, 288, 298, 301, 317.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 99/10513 A1 (AUBURN UNIVERSITY) 04 March 1999 (04.03.1999), page 13, line 20, to page 14, line 22; page 15, line 10, to page 16, line 33; page 29, line 19, to page 33, line 19; page 51, line 13, to page 58, line 34; page 63, line 25, to page 64, line 34; claims 97-99, 101-103, 105, 108-113, 180-182, and 184-186.	1, 3-4, 8-11, 19, 22, 26, 28, 30, 33-35 ---------- 2, 5-7, 17-18, 21, 23-25, 36-37
X	WO 00/03012 A2 (CALGENE LLC) 20 January 2000 (20.01.2000), page 22, line 19, to page 24, line 18; page 37, line 10, to page 43, line 32; and claims 11-12	1, 17, 26, 28, 30, 33-35
X	US 6,004,782 A (DANIELL et al) 21 December 1999 (21.12.1999), column 8, lines 23-35; column 14, line 57, to column 15, line 29; column 15, lines 43-53; column 19, line 47, to column 21, line 6.	9-11, 26
X	US 4,616,078 A (DIMARCHI) 07 October 1986 (07.10.1986), column 5, line 10, to column 7, line 45.	28, 30

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 November 2001 (01.11.2001)	03 JAN 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Anne R. Kubelik Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/06288

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BOSTON et al. Molecular chaperones and protein folding in plants. Plant Mol. Biol. 1996, Vol. 32, pages 191-222, see pages 207 and 209.	36
Y	KOTA et al. Overexpression of the Bacillis thuringiensis (Bt) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects. Proc. Natl. Acad. Sci. USA, March 1999, Vol. 96, pages 1840-1845, see pages 1841-1842.	5
Y	CRICKMORE et al. Revision of the Nomenclature for Bacillus thuringiensis Pesticidal Crystal Proteins. Micro. Mol. Biol. Rev. September 1998, Vol. 62, No. 3, pages 807-813, see Table 1.	5
Y	US 4,956,282 A (GOODMAN et al) 11 September 1990 (11.09.1990), column 3, lines 11-57.	17, 33, 37
Y	WO 99/18225 A1 (LOMA LINDA UNIVERSITY) 15 April 1999 (15.04.1999), pg. 32-33, claims 1-40.	23-25, 33
Y	SIJMONS et al. Production of correctly processed human serum albumin in transgenic plants. Biotechnology. March 1990, Vol. 8, No. 3, pages 217-221, see pages 217-218	19, 22, 33
Y	US 4,963,665 A (ROTWEIN et al) 16 October 1990 (16.10.1990), column 6, lines 58-64, Fig. 2.	18, 21, 33, 37

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/06288

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claim Nos.: 12-16, 20, 27, 29 and 31-32
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/06288

Continuation of Item 4 of the first sheet:
The title is deficient under PCT rule 4.3 because it is unrelated to the invention. The new title is

Plastid transformation vectors for expressing human proteins in plants

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s)1-4, 6-8 and 33, drawn to a plastid transformation vector comprising a proinsulin fusion protein gene.

Group II, claim(s) 5, drawn to a plastid transformation vector comprising a Cry2aA2 operon.

Group III, claim(s) 9-11 and 26, drawn to a plastid transformation vector comprising a gene for a protein based polymer fused to a biologically active molecule.

Group IV, claim(s) 17, 33 and 37, drawn to a plastid transformation vector encoding an interferon

Group V, claim(s) 18, 21, 33 and 37, drawn to a plastid transformation vector encoding an insulin-like growth factor

Group VI, claim(s) 19, 22 and 33, drawn to a plastid transformation vector encoding human serum albumin.

Group VII, claim(s) 23-25 and 33, drawn to a plastid transformation vector encoding cholera toxin B-subunit and an edible plant transformed with that plasmid.

Group VIII, claim(s) 28 and 30, drawn to a process for recovering a biopolymer by using the reversible property of the biopolymer.

Group IX, claim(s) 33-35, drawn to a plastid transformation vector encoding insulin.

Group X, claim(s) 36, drawn to a plastid transformation vector encoding a chaperonin and a promoter fusion protein.

Claims 33 and 37 will be examined to the extent they read on the elected invention.

The inventions listed as Groups I-X do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: they do not share a special technical feature. The process of recovering a biopolymer of Group VIII is unrelated to the plastid transformation vectors of Groups I-VII and IX-X. The process of recovering a biopolymer of Group VIII makes no use of the plastid transformation vectors of Groups I-VII and IX-X nor of the plants of Group VII.

Groups I-VII and IX-X share the technical feature that they are plastid transformation vectors encoding a protein. However, this technical feature is not special because it does not constitute an advance over the prior art. MCBRIDE et al (WO 00/03012, 20 January 2000) teach plastid transformation vectors encoding the biopharmaceutical proteins human growth hormone and aprotinin (pg 37-43). Thus, the technical feature is not special.

The vectors of each Group are unrelated to the vectors of each other Group because the vectors of each Group encode unrelated proteins.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/06288

Continuation of B. FIELDS SEARCHED Item 3:
AGRICOLA, BIOSIS, CAPLUS, CABA, USPAT, EPO, JPO, DERWENT
Search terms: (plastid or chloroplast) transform?, insulin, human albumin, insulin-like growth factor, interferon, polymer, GVGVP, Cry2aA2, cholera toxin, chaperonin

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 October 2001 (04.10.2001)

PCT

(10) International Publication Number
WO 01/72959 A2

(51) International Patent Classification[7]: C12N

(21) International Application Number: PCT/US01/06288

(22) International Filing Date: 28 February 2001 (28.02.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/185,987	1 March 2000 (01.03.2000)	US
60/263,424	23 January 2001 (23.01.2001)	US
60/263,473	23 January 2001 (23.01.2001)	US
60/263,668	23 January 2001 (23.01.2001)	US
Not furnished	23 February 2001 (23.02.2001)	US

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(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **DANIELL, Henry** [US/US]; 1255 Marina Point #315, Casselberry, FL 32707 (US).

(74) Agents: **WEISER, Gerard, J.** et al.; 1600 Market Street, Suite 3600, Philadelphia, PA 19103-7286 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

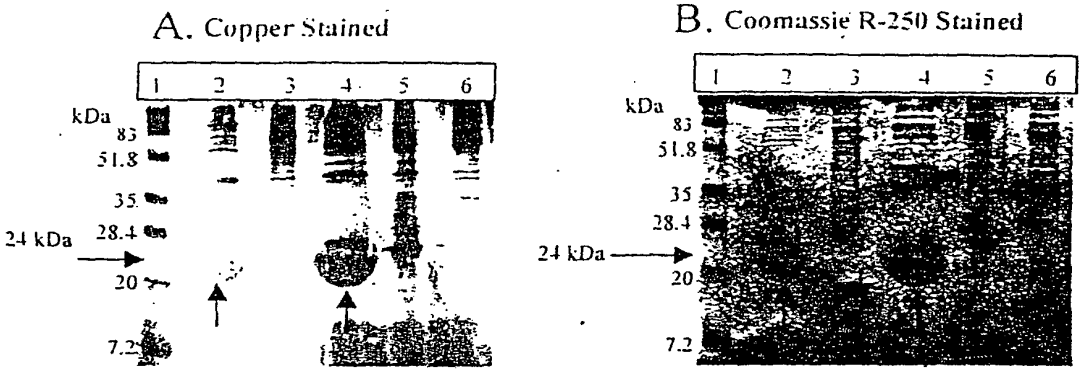
Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PHARMACEUTICAL PROTEINS, HUMAN THERAPEUTICS, HUMAN SERUM ALBUMIN, INSULIN, NATIVE CHOLERA TOXIC B SUBMITTED ON TRANSGENICS PLASTIDS

(57) Abstract: Transgenic chloroplast technology could provide a viable solution to the production of Insulin-like Growth Factor I (IGF-I), Human Serum Albumin (HAS), or interferons (IFN) because of hyper-expression capabilities, ability to fold and process eukaryotic proteins with disulfide bridges (thereby eliminating the need for expensive post-purification processing). Tobacco is an ideal choice because of its large biomass, ease of scale-up (million seeds per plant), genetic manipulation and impending need to explore alternate uses for this hazardous crop. Therefore, all three human proteins will be expressed as follows: a) develop recombinant DNA vectors for enhanced expression via tobacco chloroplast genomes; b) generate transgenic plants; c) characterize transgenic expression of proteins or fusion proteins using molecular and biochemical methods; d) large scale purification of therapeutic proteins from transgenic tobacco and comparison of current purification / processing methods in *E.coli* or yeast; e) Characterization and comparison of therapeutic proteins (yield, purity, functionality) produced in yeast or *E.coli* with transgenic tobacco; f) animal testing and pre-clinical trials for effectiveness of the therapeutic proteins.

Figure 1: Biopolymer-Proinsulin Fusion Protein Expression



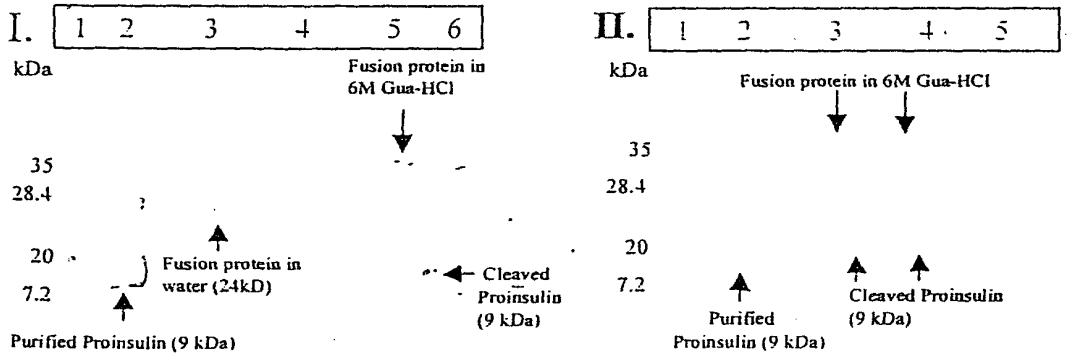
PAGE: 15% Glycine large gel

A. Copper Stained: Gel rinsed in water for 10 min, stained with 0.3M $CuCl_2$ for 5min, and rinsed in water for 3min.

B. Coomassie R-250 Stained: The same gel was first rinsed for 20min in water and then stained for 1hr, and destained overnight.

A. and B. Lanes. 1, Prestained Marker (BioRad) ; 2. Sonic extract of pSBL-OC-XaPris ; 3, reverse orientation of fusion protein of pSBL-OC- XaPris: 4, Sonic extract of pLD-OC-XaPris: 5, inverse orientation of pLD-OC- XaPris; 6, Sonic extract of *E. coli* strain XL-1 Blue containing no plasmid .

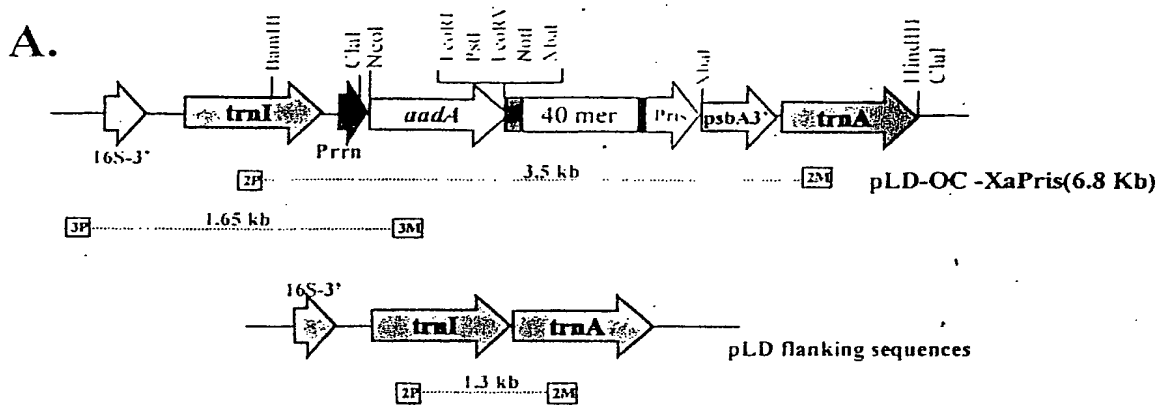
C. Western Blot of Biopolymer-Proinsulin Fusion Protein



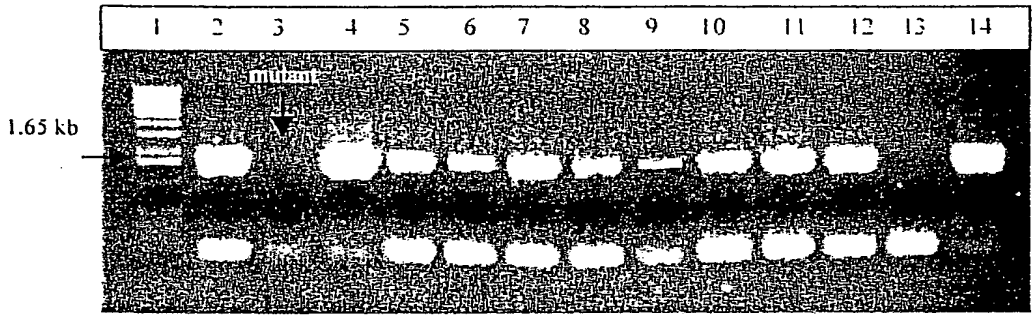
I. Lanes. 1, BioRad Prestained Marker; 2, 3ug of Purified Human Proinsulin; 3, 5ug of pSBL-OC-XaPris (sonication and purification of biopolymer twice); 4, Negative control, XL-1 Blue *E.coli*; 5, Sonic extract pSBL expressing cells (6M Guanidine Hydrochloride Phosphate Buffer, pH 7.0); 6, Sonic extract of XL-1 Blue *E.coli* with no pSBL.

II. Lanes. 1, BioRad Prestained Marker; 2, 5ug of Purified Human Proinsulin: 3, Sonic extract of pSBL-OC-XaPris expressing cells (6M Guanidine Hydrochloride Phosphate Buffer. pH 7.0): 4. Sonic extract of pLD-OC-XaPris expressing cells (Gua-HCl); 5, Sonic extract of XL-1 Blue *E.coli* with no plasmid.

Figure 2: Confirmation of Chloroplast Integration by PCR of Polymer-Proinsulin Fusion Gene

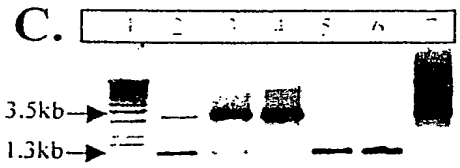


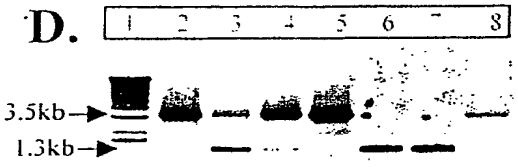
B. Confirmation of *aadA* integration into the chloroplast genome - Primers: **3P/3M**



A. Lanes, 1, 1 kb marker; 2. clone L19b (L=pLD-OC -XaPris) vector0; 3. clone L9 (mutant); 4. L1; 5, L8d; 6, L10a; 7. S30b (S=pSBL-OC -XaPris vector); 8. S20a; 9. S60; 10, S7a; 11. S28; 12, S41b; 13, Petit havana (not transgenic):14, Positive control (BADH gene present in chloroplasts from transgenic plants already confirmed)

Confirmation of integration of *aadA* and biopolymer-proinsulin fusion genes into the chloroplast genome - Primers: **2P/2M**



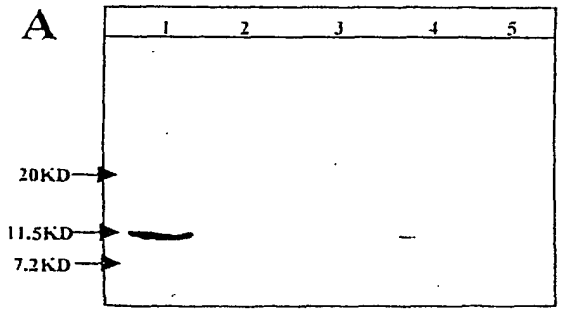


PCR of pLD clones: Lanes, 1, 1kb marker; 2, L17a; 3, L19b; 4, L8d; 5, L9; 6, Petit havana (not transgenic); 7, pLD vector as positive control

PCR of pSBL clones: Lanes, 1, 1kb marker; 2, S17a; 3, S30b; 4, S7a; 5, S41b;6, L9(mutant) 7, Petit havana (not transgenic); 8, pSBL vector as positive control

Figure 3 : CTB Gene Expression and Chloroplast Integration

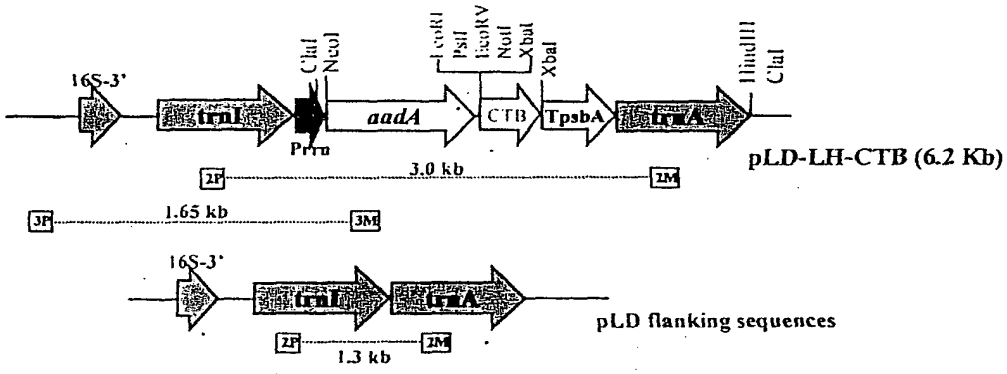
A



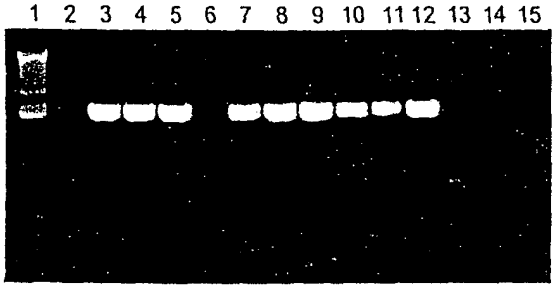
Western Blot analysis of CTB expression in *E.coli* (15% PAGE):

Lane 1: Purified bacterial CTB (0.5µg) ; 2 & 4: Transformed E.coli culture-24 h and 48 h resply. ; 3 & 5 : Untransformed E.coli culture- 24 h and 48 h resply.

B

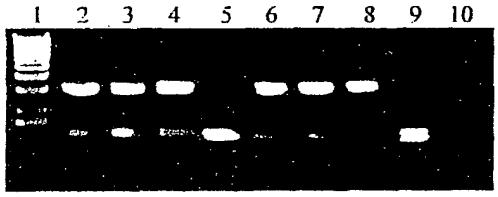


C. PCR confirmation of *aad*A gene integration into chloroplast genome -3P/3M primers



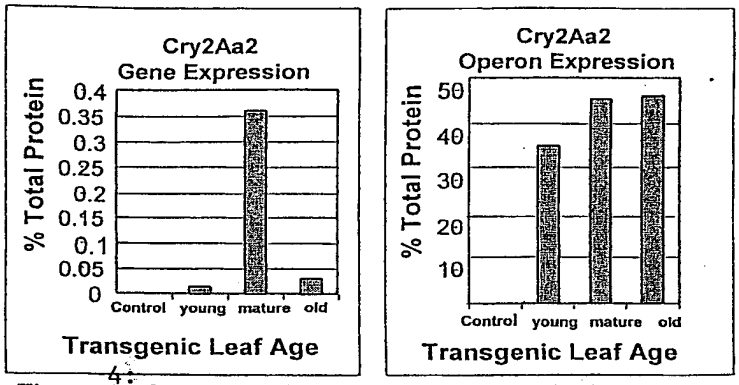
PCR of clones of 1st. round of selection : Lane 1:1 Kb marker ; 2 - 12 : Plant total DNA from spec.r clones 1-11 (Note: Lanes 2 & 6 are mutants); 13: Untransformed plant; 14: pLD-LH-CTB vector ; 15: No DNA template.

D. PCR confirmation of integration of *aad*A and CTB gene into chloroplast genome - 2P/2M primers



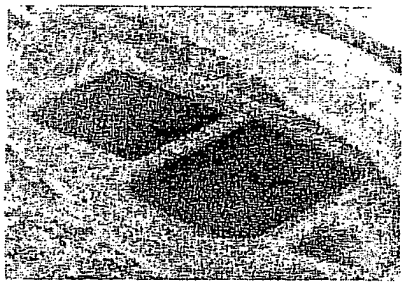
PCR of clones of 2nd. round of selection : Lane 1: 1 Kb marker; 2 - 7 : Plant total DNA from spec.r clones 1- 6 (Note: Lane 5 is a mutant); 8: pLD-LH-CTB vector; 9: Untransformed plant ; 10 : No DNA template.

• **Expression of bacterial operon in transgenic chloroplasts.**



4.

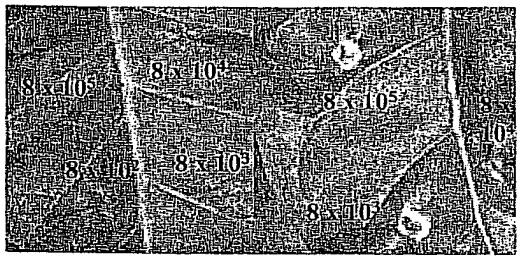
Figure 1: Cry2A protein concentration determined by ELISA in transgenic leaves. Note 100-fold increase in protein accumulation in the presence of the putative chaperonin, ORF2.



5.

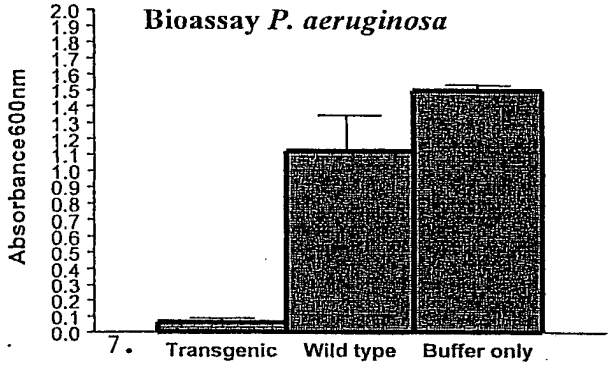
Figure 2 :Inmunogold labeled electron microscopy of mature transgenic leaf. Cry2Aa2 crystals in a transgenic chloroplast expressing the cry2A operon.

• **Expression of a small (22aa) peptide in transgenic chloroplasts.**



6.

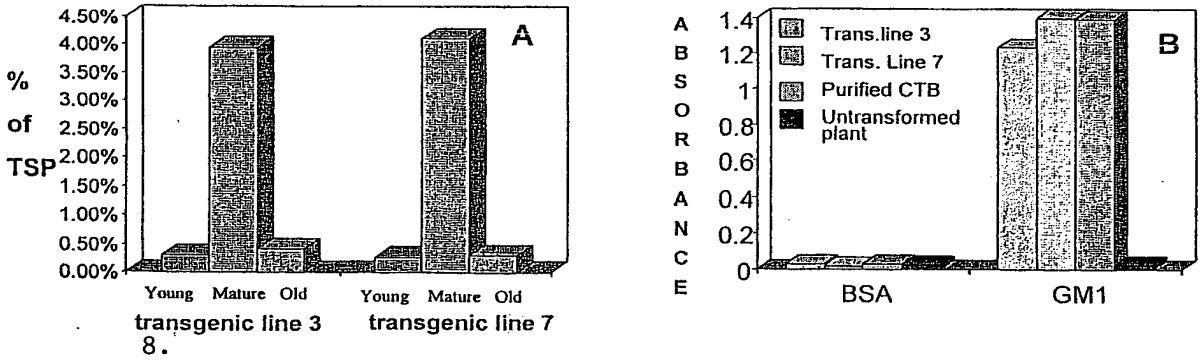
Figure 3 . Leaves were infected with 10 µl of $8x10^5$, $8x10^4$, $8x10^3$ and $8x10^2$ cells of *P. syringae*. Photos were taken 5 days after inoculation. 1-2 µg of antimicrobial peptide (AMP) is required to kill 1000 bacterial cells. Local concentration at the site of infection is estimated to be 200-800µg AMP.



7.

Figure 4. Total plant protein was mixed with 5µl of mid-log phase bacteria from overnight culture, incubated for 2 hours at 25°C at 125rpm and grown in LB broth overnight. Based on minimum inhibitory concentration of 1-2 µg AMP/1000 bacterial cells, the expression level was calculated to be 21.5-43% of the total soluble protein.

• **Expression of Oligomeric form (disulfide bonded) CTB in transgenic chloroplasts.**



8.

Figure 5: A) CTB ELISA quatification is shown as a percentage of the total soluble plant protein. Total soluble plant protein from young, mature and old leaves of transgenic lines 3 and 7 was quantified. **B) CTB-GM1 Ganglioside binding ELISA** assays: Plates coated first with GM1 gangliosides and BSA were plated with total soluble plant protein from lines 3 and 7, untransformed plant total soluble protein and purified bacterial CTB. The absorbance or the GM1 ganglioside-CTB antibody complex was measured.

• **Expression of CTB oligomers.**

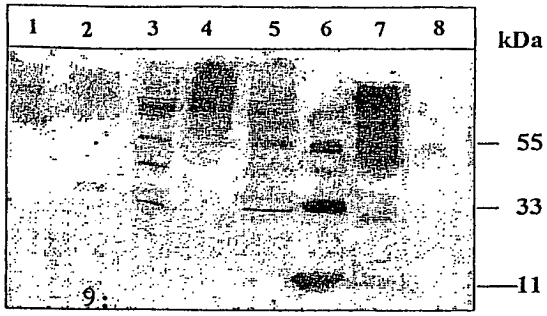


Figure 6: 12% reducing PAGE. Chemiluminescent detection with rabbit anti-cholera serum (1^0) and AP labeled mouse anti-rabbit IgG (2^0) antibodies. Untransformed, boiled(1) and unboiled (2); Transformed, boiled (3&5) and unboiled (4);Purified CTB boiled (6)and unboiled (7); Marker (8).

• **Expression & assembly of disulfide bonded Guy's 13 monoclonal antibody .**

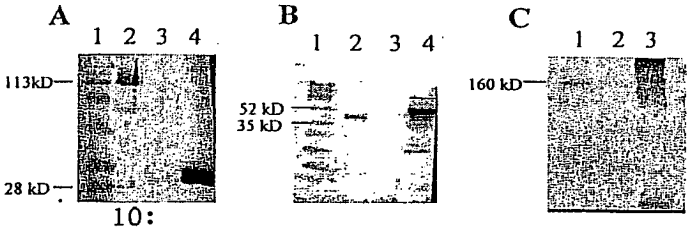


Figure7: A, B) reducing gels. 1:markers, 2:Transgenic extract showing expression of light (A) and heavy chain (B) in chloroplasts, 3: Untransformed, 4: Human IgA. C) non-reducing gel. 1.Transgenic extract showing assembly, 2: Untransformed, 3: Human IgA. Blots A & C were detected with AP conjugated goat anti-human kappa antibody. Blot B was detected with AP conjugated goat anti-human IgA antibody.

• **Marker-free chloroplast transgenic plants.**

Selectable marker	Plate No.	Total no. of leaf discs	No of responding leaf discs	Total no of shoots/ plate
BADH	1	3	3	43
	2	6	4	23
	3	11	9	33
	4	7	6	19
	5	6	4	16
	6	9	7	18
Spectinomycin	1	5	0	0
	2	5	0	0
	3	5	3	3
	4	5	2	2
	5	5	0	0
	6	5	1	1
	7	5	1	2
	8	5	1	2
	9	5	0	0
	10	5	0	0
Control		5	0	0

Table 1: Comparison of Spectinomycin and Betaine aldyhyde as the selectable marker for the first round of selection.

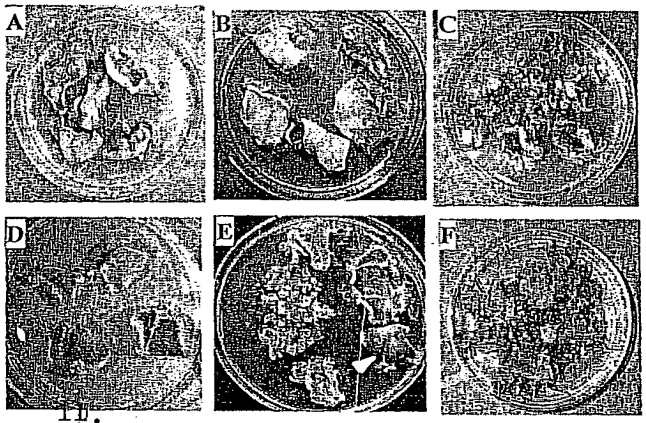


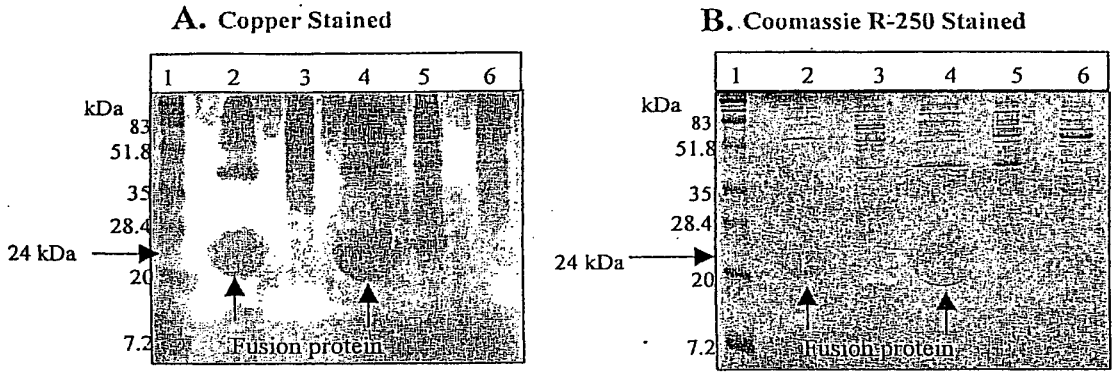
Figure 8: Comparison of betaine aldehyde and spectinomycin selection. A. *N. tabacum* Petit Havana control in RMOP medium containing spectinomycin after 45 days. B. Bombarded leaf discs selected on spectinomycin in RMOP medium after 45 days. C. Spectinomycin resistant clones cultured again (second round) to obtain homoplasmy. D. Petit Havana control in RMOP medium containing Betaine Aldehyde after 12 days of culture. E. Bombarded leaf discs selected on Betaine Aldehyde in RMOP medium after 12 days of culture; arrow indicates unbombarded leaf disc as control. Note that 23 shoots are formed on a disc selected on betaine aldehyde against 1-2 shoots per disc on spectinomycin. F. Betaine aldehyde resistant clones cultured again (second round) to obtain homoplasmy.

• **Codon composition and expression levels.**

Open reading Frame	%TSP	%A+T	%psbA	%cp tRNA
Plastid miniproinsulin	?	66	100	62
CTB	4.1	66	47	34
Cry2A operon	47	65	37	37
Plastid proinsulin	?	64	100	49
Antimicrobial peptide	21	63	35	35
Guy's light chain	<1%	49	31	44
Optimized biopolymer	?	47	100	40
Guy's heavy chain	<1%	40	25	44
Human proinsulin	?	38	26	44

Table 2 (Left): Black indicates genes with unmodified native codon composition and their expression levels observed in transgenic chloroplasts, ranked by AT% in ascending order. Red indicates genes to be investigated. Kusnadi et al. (1997) suggest that a minimum of 1% TSP is adequate for commercial feasability. See section d) for details of AT content, %psbA optimal codons and % of codons that match the cp tRNA pool. TSP: % total soluble protein

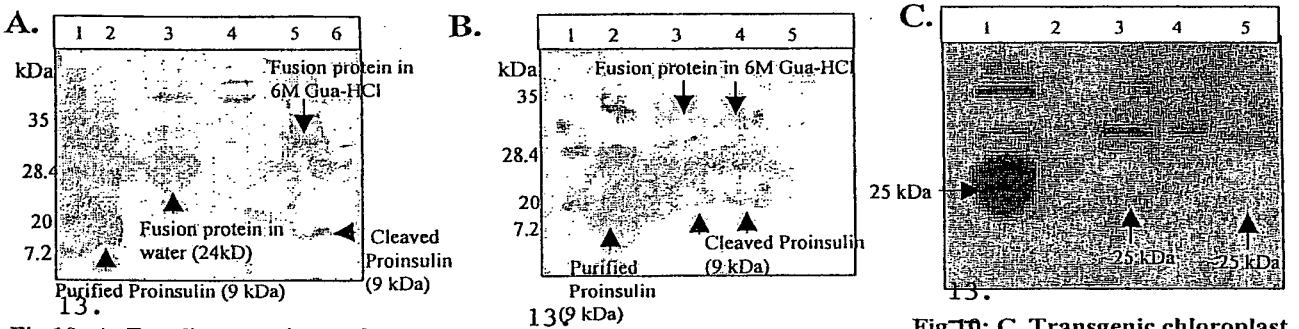
Biopolymer-Proinsulin Fusion Protein Expression



12.

Fig 9, A and B Lanes: 1, Prestained Marker (BioRad) ; 2, Sonic extracts of pSBL-OC-XaPris ; 3, reverse orientation of insert in pSBL-OC- XaPris; 4, pLD-OC-XaPris; 5, reverse orientation of pLD-OC- XaPris; 6, *E. coli* XL-1 Blue cells with no plasmid .

Western Blots of Biopolymer-Proinsulin Fusion Protein After Single Step purification



13.

Fig 10: A. E. coli expression and cleavage Lanes: 1, BioRad Prestained Marker; 2, 3ug of Purified Human Proinsulin; 3, 5ug of pSBL-OC-XaPris; 4, Negative control, reverse orientation; 5, pSBL expressing cells (6M Guanidine Hydrochloride Phosphate Buffer, ph7.0); 6, XL-1 Blue *E.coli* with no pSBL.

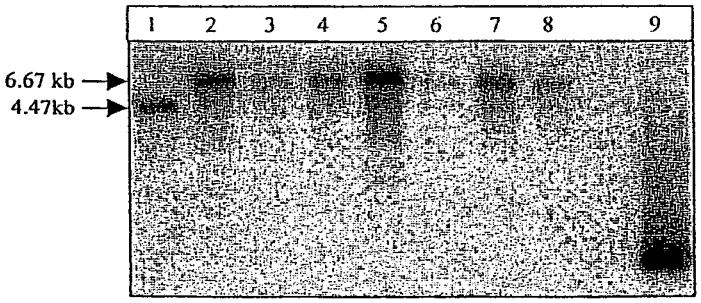
13(9 kDa)

Fig 10: B. E. coli expression and cleavage Lanes: 1, BioRad Prestained Marker; 2, 5ug of Purified Human Proinsulin; 3, pSBL-OC-XaPris (6M Guanidine Hydrochloride Phosphate Buffer, ph7.0); 4, pLD- OC-XaPris; 5, XL-1 Blue *E.coli* with no plasmid.

13.

Fig 10: C. Transgenic chloroplast expression Lanes: 1, Purified *E. coli* protein from pLD-OC-XaPris expression; 2, negative control (Petit Havana); 3-5, Chloroplast transgenic lines. Note dimer, tetramer and hexamer aggregates of polymer-insulin fusion protein

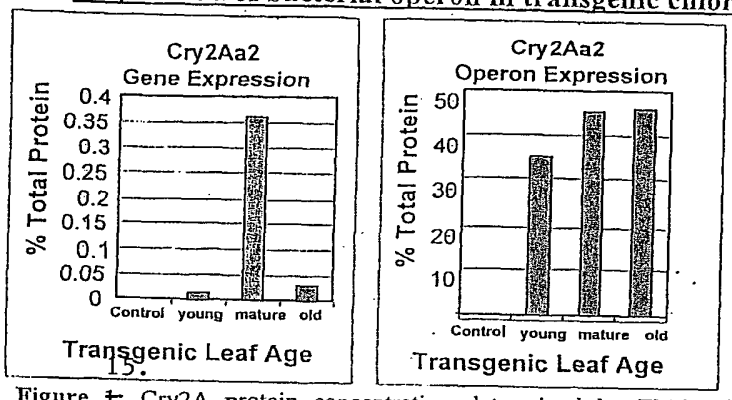
Confirmation of chloroplast integration and homoplasmy/heteroplasmy by Southern Blot Analysis



14.

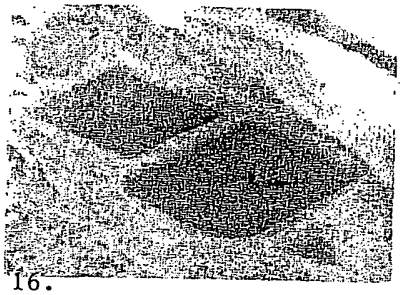
Fig 11. Biopolymer-proinsulin fusion gene integration into the chloroplast genome confirmed by Southern blot analysis. Lanes: 1, Petit Havana (negative control); 2-5, pLD-OC-XaPris clones T_0; 6-8, pSBL-OC-XaPris clones T_0; 9, probe(positive control). Homoplasmy is seen in most transgenic lines while a few transgenic lines show heteroplasmy.

• Expression of bacterial operon in transgenic chloroplasts.



15.

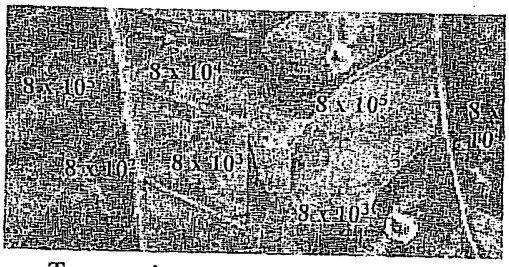
Figure 1: Cry2A protein concentration determined by ELISA in transgenic leaves. Note 100-fold increase in protein accumulation in the presence of the putative chaperonin, ORF2.



16.

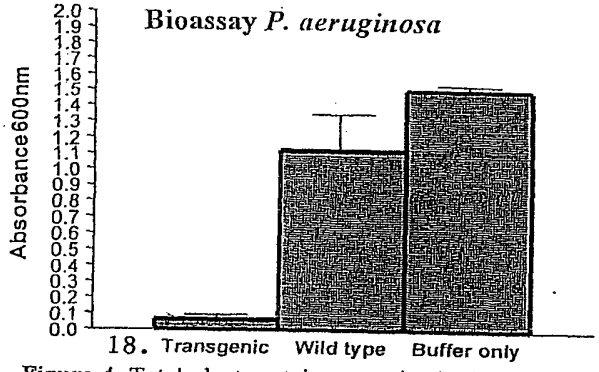
Figure 2: Inmunogold labeled electron microscopy of mature transgenic leaf. Cry2Aa2 crystals in a transgenic chloroplast expressing the cry2A operon.

• Expression of a small (22aa) peptide in transgenic chloroplasts.



17.

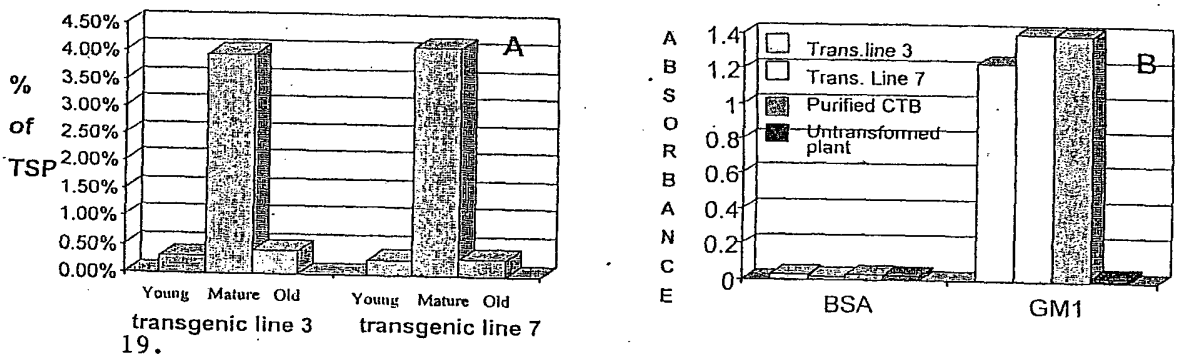
Figure 3: Leaves were infected with 10 µl of $8x10^5$, $8x10^4$, $8x10^3$ and $8x10^2$ cells of *P. syringae*. Photos were taken 5 days after inoculation. 1-2 µg of antimicrobial peptide (AMP) is required to kill 1000 bacterial cells. Local concentration at the site of infection is estimated to be 200-800µg AMP.



18.

Figure 4: Total plant protein was mixed with 5µl of mid-log phase bacteria from overnight culture, incubated for 2 hours at 25°C at 125rpm and grown in LB broth overnight. Based on minimum inhibitory concentration of 1-2 µg AMP/1000 bacterial cells, the expression level was calculated to be 21.5-43% of the total soluble protein.

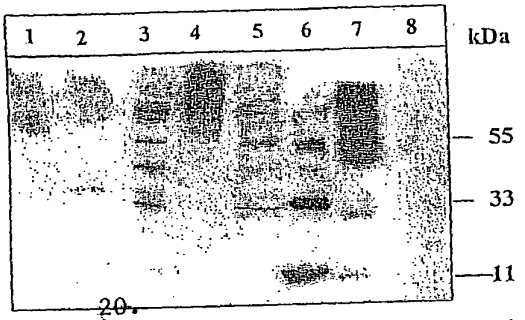
• Expression of Oligomeric form (disulfide bonded) CTB in transgenic chloroplasts.



19.

Figure 5: A) CTB ELISA quatification is shown as a percentage of the total soluble plant protein. Total soluble plant protein from young, mature and old leaves of transgenic lines 3 and 7 was quantified. B) CTB-GM1 Ganglioside binding ELISA assays: Plates coated first with GM1 gangliosides and BSA were plated with total soluble plant protein from lines 3 and 7, untransformed plant total soluble protein and purified bacterial CTB. The absorbance or the GM1 ganglioside-CTB antibody complex was measured.

• Expression of CTB oligomers.



20.

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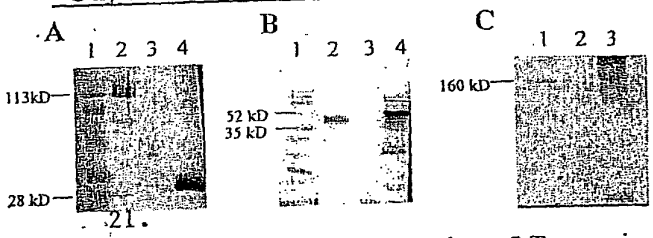
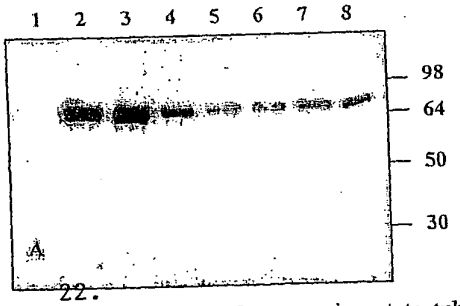
• Expression & assembly of disulfide bonded Guy's 13 monoclonal antibody .



21.

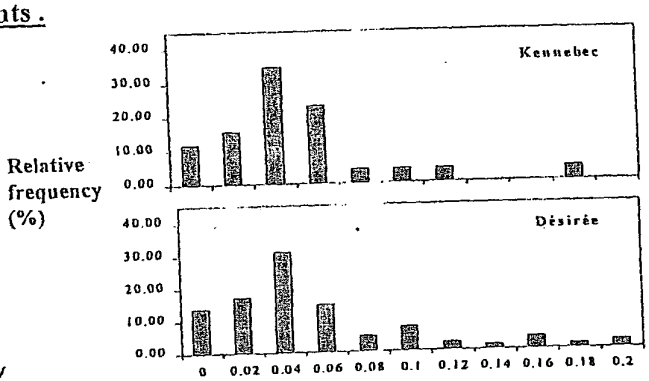
Figure 7: A, B) reducing gels. 1:markers, 2:Transgenic extract showing expression of light (A) and heavy chain (B) in chloroplasts, 3: Untransformed, 4: Human IgA. C) non-reducing gel. 1.Transgenic extract showing assembly, 2: Untransformed, 3: Human IgA. Blots A & C were detected with AP conjugated goat anti-human kappa antibody. Blot B was detected with AP conjugated goat anti-human IgA antibody.

• HSA Nuclear transformation of potato plants .



22.

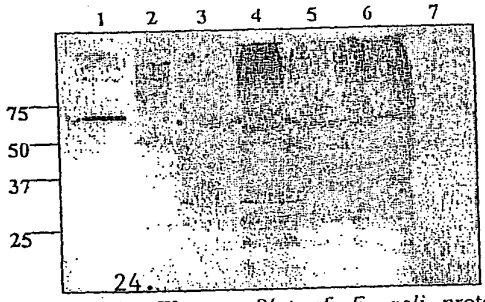
Figure 8: *Western Blot* of transgenic potato tubers, cv Desiree. 30 µg of tuber protein was loaded per lane and probed with anti-HSA antibody. 1: wild type; 2: 40 ng of pure HSA; 3-8:diferent trangenic lines, showing different levels of expression.



23.

Figure 9: Frecuency histogram including percentage Kennebec and Désirée transgenic plants expressing different HSA levels. Results are shown as the percentages of transgenic plants (vertical axis) that express a specific level of HSA of the total soluble protein (horizontal axis).

• Expression of HSA by chloroplast vectors in *E. coli.*



24.

Figura 10: *Western Blot* of *E. coli* protein extracts. 1: 50 ng pure HSA; 2: molecular weigh marker; 3: pLD-HSA (control without RBS); 4: PLD- 5'UTR-HSA; 5: pLD-RBS-HSA; 6: pLD-ORF1+2-HSA; 7: *E. coli* without pLD vector.

• Codon composition and expression levels.

Open reading Frame	% TSP	% A+T	% psbA	%cp tRNA
CTB	4	66	47	34
Cry2A operon	47	65	37	37
Antimicrobial peptide	21-43	63	35	35
HSA	?	57	57	47
Interferon alpha	?	54	31	40
RUBISCOssTP	?	50	32	42
Guy's light chain	<1%	49	31	44
IGF-I	?	41	20	30
Guy's heavy chain	<1%	40	25	44

Table 1: Unmodified native codon composition and expression levels observed in transgenic chloroplasts. See section d) for details of AT content, %psbA optimal codons and % of codons that match the cp tRNA pool. TSP: % total soluble protein

• **Expression of HSA via chloroplast genome in tobacco.**

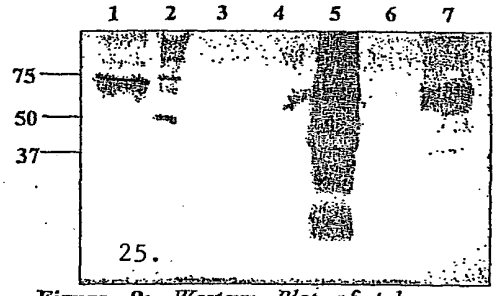
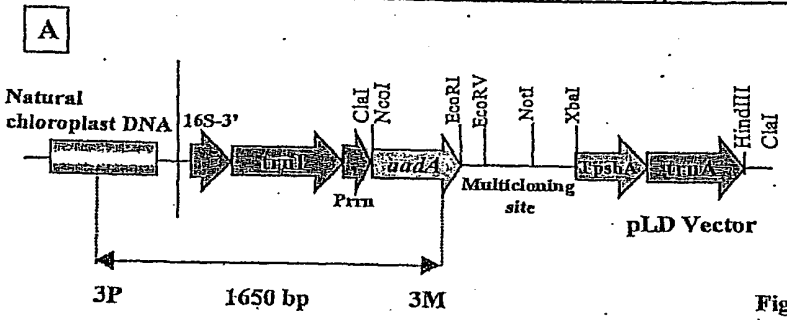


Figura 9: *Western Blot* of tobacco protein extracts. 1: 40 ng pure HSA; 2: molecular weight marker; 3 and 4: wild type plant extracts; 5: extracts form plants transformed with PLD-5'UTR-HSA; 6: pLD-RBS-HSA; 7: pLD-ORF1+2-HSA. 30 micrograms of plant protein were loaded per well.

•**PCR analysis of transformants to determine integration of HSA gene into the chloroplast genome.**



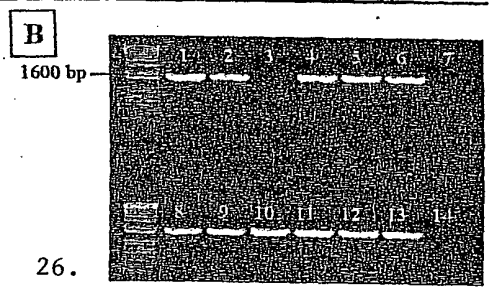
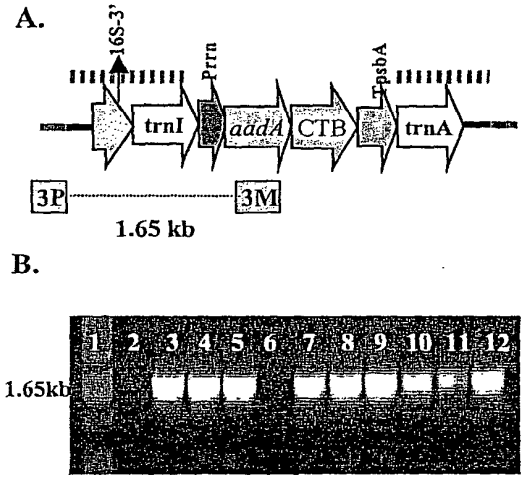


Figura 10: A) Map of the pLD chloroplast transformation vector and primer landing sites. B) Agarose gel containing PCR products using total plant DNA as template from plants transformed with: 1,2,3: pLD-RBS-HSA; 4,5,6: pLD-5'UTR-HSA; 8,9,10: pLD-ORF1+2-HSA; 11,12,13: pLD-ORF1+2-5'UTR-HSA; 7,14: negative controls (from untransformed plants); 3: mutant.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Prrn
TpsbA
trnI
aadA
CTB
trnA
3P
3M
1.65 kb
B.
1.65kb
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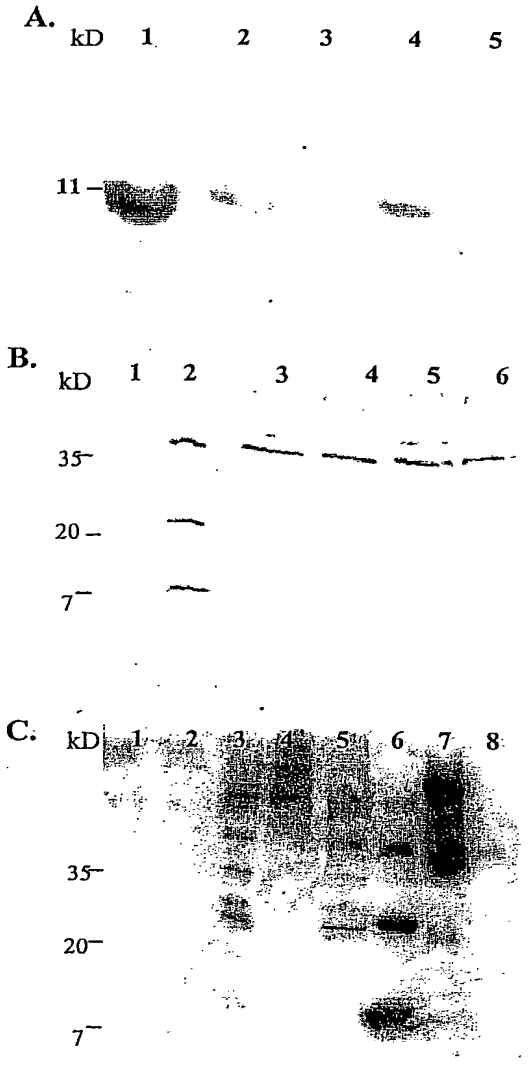
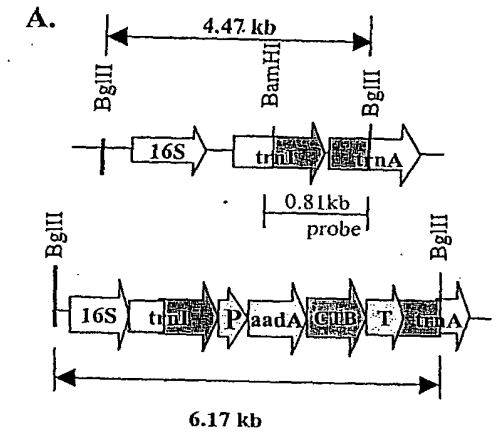
27.

Figure 1



28.

Figure 2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BglII
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trnI
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probe
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BglII
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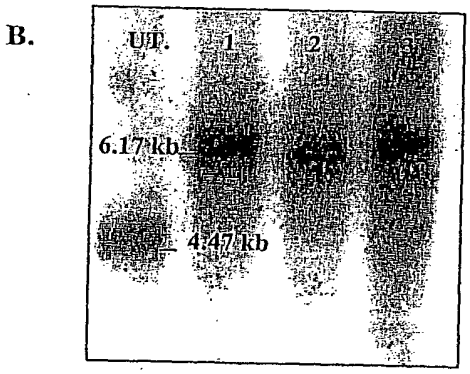
P
aadA
GUB
T
trnA
6.17 kb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2
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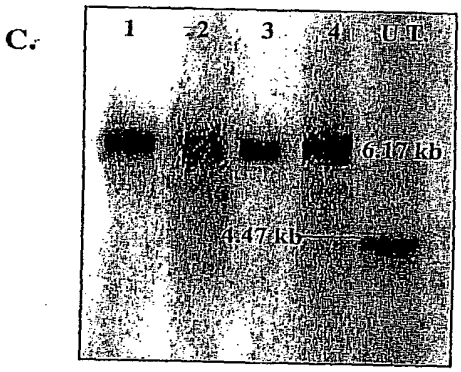
C.
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6.17 kb
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Figure 3

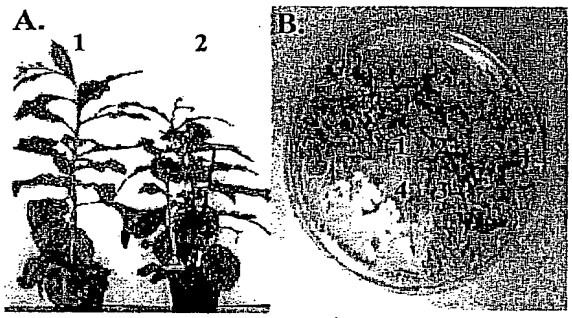


Figure 4

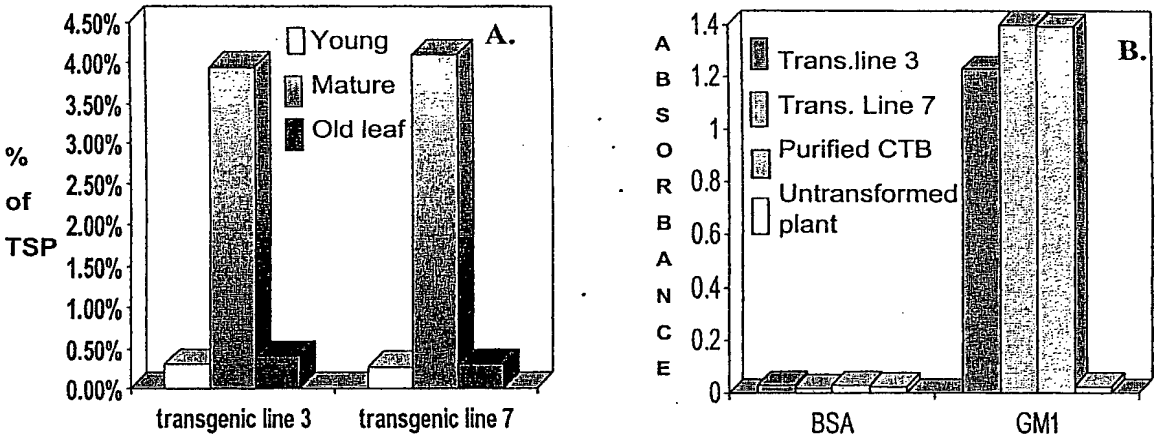
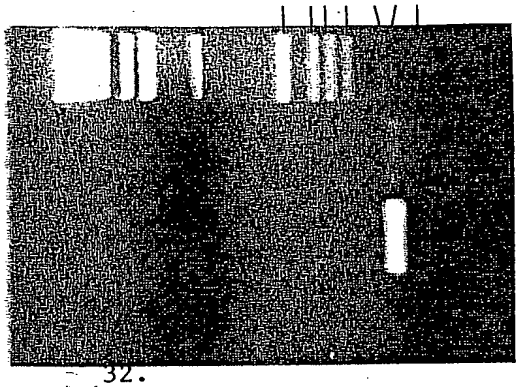

A.
% of TSP
4.50%
4.00%
3.50%
3.00%
2.50%
2.00%
1.50%
1.00%
0.50%
0.00%
Young
Mature
Old leaf
transgenic line 3
transgenic line 7
B.
ABSORBANCE
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Trans.line 3
Trans. Line 7
Purified CTB
Untransformed plant
BSA
GM1

Figure 5



32.

ure 1 (above): cloning of the PsbA 5' untranslated
on (5'UTR) from the chlorplast genome

34

ure 3 (below): a comparisison of the DNA sequences of native
ıan proinsulin (top) and plastid modified proinsulin (bottom)

```
gtgaaccaacacctgtgcggctcacacctggtggaagctctctacctagtgtgcggg
 || |||||||||  | ||  ||  ||  |||||  ||  ||||||  |  |||  ||||  ||  ||
gtaaaccaacacttatgtggttctcacctagtagaagctttatacttagtatgtggt

ıcgaggcttcttctacacacccaagacccgccgggaggcagaggacctgcaggtgggg
|||  ||  ||||||||||||  ||  ||  ||  ||  ||  ||  ||  ||  |  ||  ||  ||
ıcgtggtttcttctacactcctaaaactcgtcgtgaagctgaagatttacaagtaggt

ggtggagctgggcgggggccctggtgcaggcagcctgcagcccttggccctggagggg
 ||  ||  |  ||  ||  ||  ||||||||  ||  |  ||  ||  ||  ||  |  ||  ||
agtagaattaggtggtggtcctggtgctggttctttacaaccttagctttagaaggt

cctgcagaagcgtggcattgtggaacaatgctgtaccagcatctgctccctctaccag
  |  ||  ||  |||||  |||||  ||||||||  |||||  ||  ||  ||  |  ||||||
tttacaaaaacgtggtattgtagaacaatgttgtacttctatttgttctttataccaa
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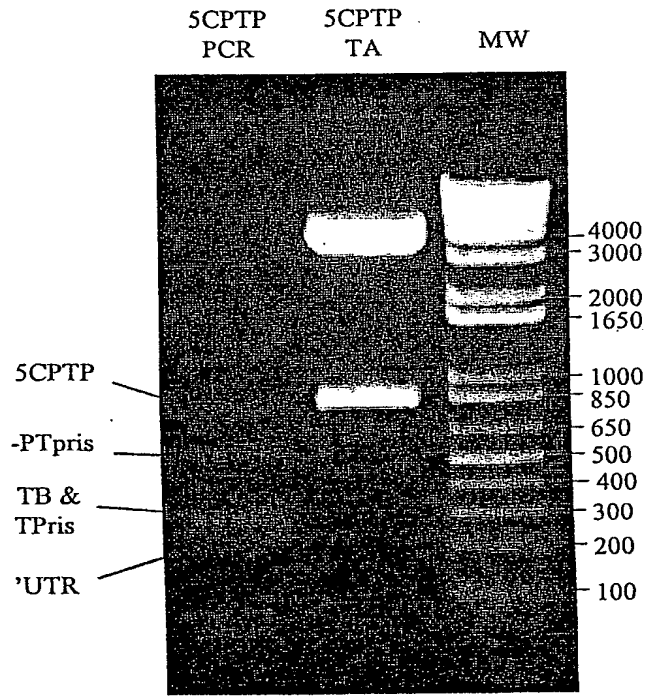
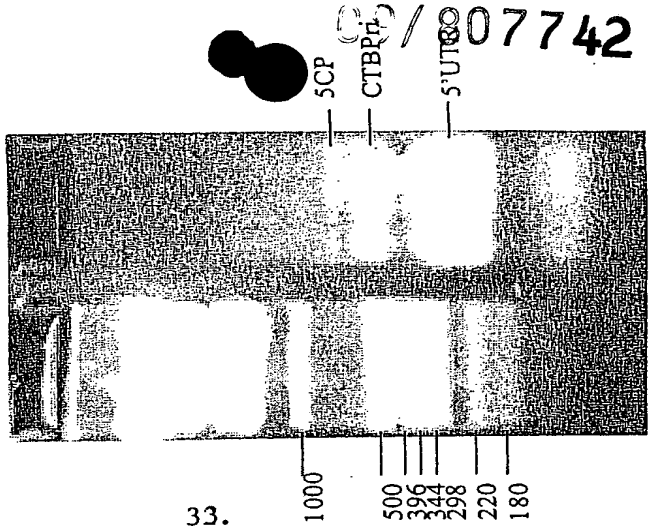
ggagaactactgcaacta Native Human Proinsulin
|| |||||||| |||||
agaaaactactgtaacta Chloroplast Modified Proinsulin



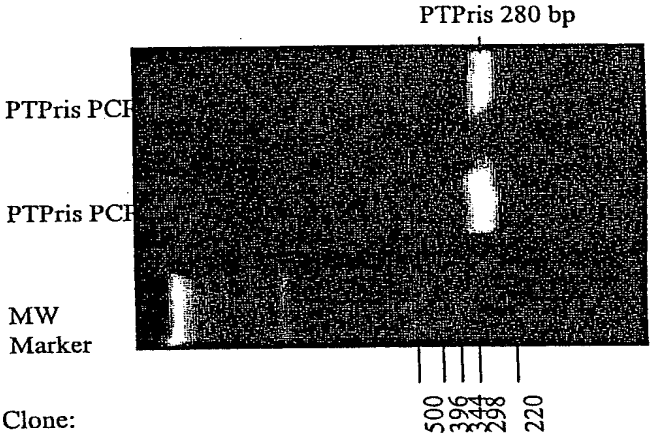


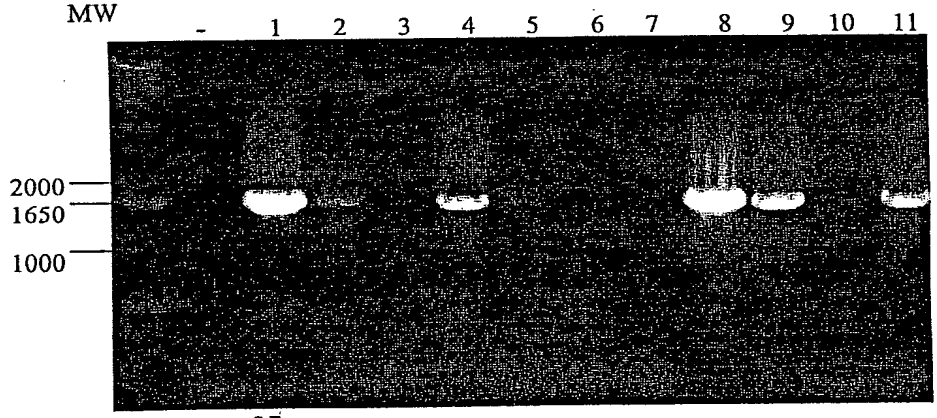
33.

Figure 2 (above): SOEing of the 5'UTR to the CTB- human proinsulin sequence. **5CP** is the PSbA 5'UTR and the Cholera Toxin B subunit (CTB) human proinsulin fusion

35

Figure 4 (below): Recursive PCR to synthesize the chloroplast modified proinsulin (Ptpris)





37.

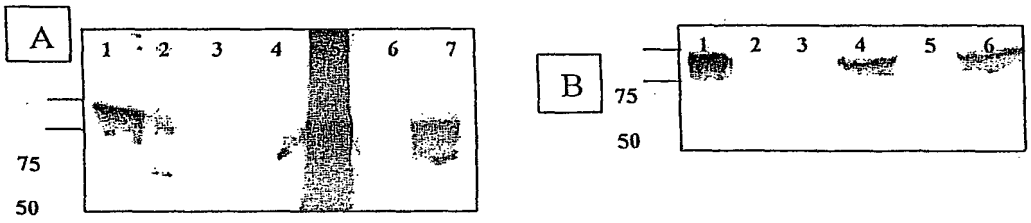
Figure 6 (above): PCR products to confirm construct integration into the chloroplast genome using two primers, 3P and 3M. 3P anneals to the native chloroplast genome and 3M anneals to the introduced spectinomycin resistance gene, *aadA*, creating a 1600 bp product only in transgenic clones

36.

Figure 5 (left): SOEing of the 5'UTR, CTB, and plastid modified proinsulin, which results in the fusion of all three sequences denoted as **5CPTP**. The second lane show this

promoter. IFNα5 gene was cloned into the pLD using both sequences and bombarded into tobacco leaves. Shoots appeared after 5 weeks and the second round of selection is in progress.

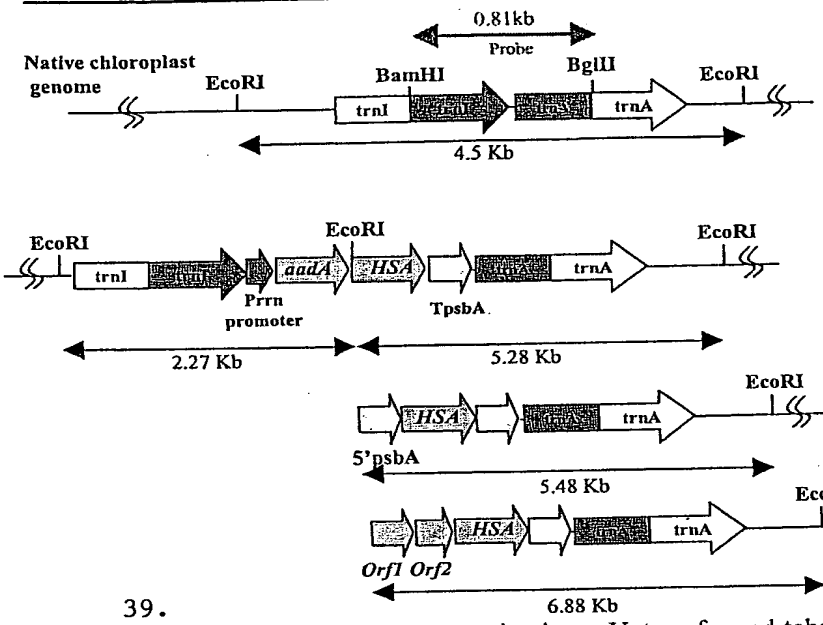
• **Expression of HSA via the chloroplast genome in tobacco.**



38.

Figure 1: *Western Blot* of tobacco protein extracts. A) 1: 40 ng pure HSA; 2: molecular weight marker; 3,4,6: untransformed plant extracts; 5: extract from plants transformed with: PLD- 5'UTR-HSA; 7: pLD-Orf1Orf2-HSA. B) 1: 40 ng pure HSA; 2: molecular weight marker; 3,5: untransformed plant extracts; 4: extract from plants transformed with: PLD- RBS-HSA; 6: pLD-Orf1Orf2-HSA. 10 micrograms of plant protein were loaded in each well.

• Southern blot analysis of HSA transgenic tobacco plants.



39.

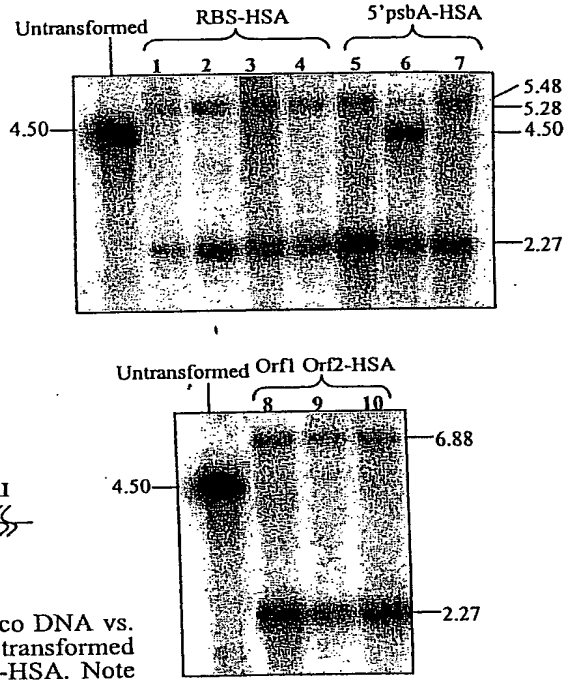
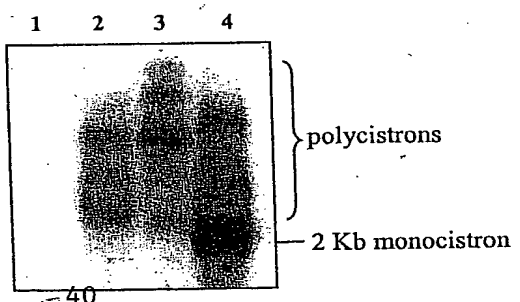


Figure 2: *Southern Blot* of HSA transgenic plants. Untransformed tobacco DNA vs. transgenic tobacco DNA digested with EcoRI. 1,2,3,4: DNA from plants transformed with pLD-RBS-HSA; 5,6,7: pLD-5'psbA-HSA; 8,9,10: pLD-Orf1-Orf2-HSA. Note homoplasmy in all the clones except number 6.

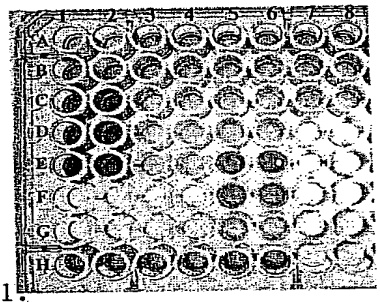
• Northern blot analysis of HSA transgenic tobacco plants.



40.

Figure 3: *Northern Blot* of HSA transgenic plants using HSA probe (1.8 kb). 1: untransformed tobacco RNA. 2: RNA from plants transformed with: pLD-RBS-HSA; 3: pLD-Orf1-Orf2-HSA; 4: pLD-5'psbA-HSA. Note different sizes of transcripts and the presence of monocistrons in number 4.

• ELISA analysis of HSA transgenic tobacco plants.



41.

Figure 4: *ELISA* of HSA transgenic plants. A-E/1-2: HSA standards; F/1-2: Blank; G/1-2: Untransformed Petit Havana protein extracts; D-E/3-4: proteins from plants transformed with pLD-Orf1Orf2-HSA; F-G/3-4 and D-H/7-8: pLD-RBS-HSA; Rest of the wells contain extracts from different clones transformed with pLD-5'psbA-HSA.

• IGF-I optimized sequence and PCR product after synthesis of the new gene.

A

```
ggaccggagacgctctgcggggctgagctggtggatgctcttcagttcgtg
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tcagct
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B

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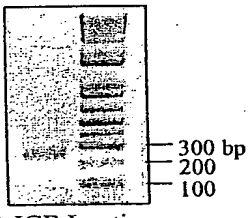
C



42.

Figure 5: A) IGF-I native sequence coding for the mature protein. B) IGF-I optimized sequence according to chloroplast preferred codon usage. Note changes in red. C) IGF-I synthetic gene after recursive PCR.